Coupled SFE/SFC/GC for the Trace Analysis of Pesticide Residues in Fatty Food Samples

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Supercritical fluid chromatography (SFC)
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Summary

An on-line SFE-chromatographic system, where SFE has been coupled with SFC and GC, was developed and utilized for trace analyses of organochlorine and organophosphorus pesticide residues from gram-sized complex sample matrices, such as chicken fat, ground beef, and lard. The SFE process and chromatographic techniques were instrumentally integrated for efficient and automated on-line analysis, having minimal sample handling between the sample preparation and separation steps. A cleanup step, incorporating packed column SFC, allowed the fractionation of relatively small-sized, non-polar pesticides from the co-extracted fatty materials. This permitted final high-resolution separation of analytes on a capillary GC column. Detection of pesticides was accomplished using selective electron-capture and nitrogen-phosphorus detectors. Pesticide concentrations determined with the on-line system were accurate and reproducible, for fatty samples containing both fortified and incurred pesticides. This method, utilizing supercritical carbon dioxide, was considerably faster and less laborious than the conventional analytical procedures based on liquid extraction.

1 Introduction

Improved analytical protocols that are rapid and require minimum effort are continually being sought to test foods for pesticide residues. In addition, increasing attention is being devoted to the development of sample preparation methods which can be integrated with the separation and detection steps, thereby permitting integration of the entire analysis scheme [1]. Such an approach will facilitate the routine analysis of large numbers of samples.

In general, a major limiting factor in pesticide residue analysis is not the detection of the compounds of concern but the tedious techniques used to isolate the chemical residues from a complex matrix. For regulatory purposes, food samples containing high level of fat are of particular concern, because of their propensity to accumulate toxicants such as organo-chlorine and -phosphorus pesticides. Accurate determination of these analytes at trace levels, requires extensive sample preparation prior to the actual analysis, in order to remove interfering compounds and to isolate the analytes [2,3].

Recently, supercritical fluids have been used as an alternative medium for the extraction and preparation of samples for residue analysis. In this case, the unique properties exhibited by supercritical fluids have provided a superior extraction technique over the conventional, liquid-based extraction. Numerous applications of SFE for the analysis of pesticide residues in food samples have been reported [4–7].

A logical extension of SFE is to combine the process, on-line with a chromatographic technique, so that sample preparation and analysis are instrumentally integrated and easily automated. On-line coupling of SFE with various types of chromatographic techniques (SFE/GC, SFE/SFC, SFE/HPLC, *etc.*) have already been achieved [4,8–12]. Although many of these on-line SFE/chromatographic systems utilize the inherent advantages of SFE, their applications are limited to the sample matrices, devoid of interfering materials that are also soluble in the supercritical fluid. For instance, SFE/GC is often impractical for the trace analysis of multiple residues in complex samples, due to the frequent contamination of the chromatographic apparatus by unwanted co-extractives [11]. This problem is significant in the direct analysis of samples of biological origin or food-related samples, which often contain a considerable amount of extractable matter. Selective SFE of the desired analytes by adjustment of the supercritical fluid density alone [13], or by adding an adsorbent to prevent extraction of the fat [14], have been studied; but such methods are complicated, and often do not yield complete separation.

Multidimensional chromatography is an alternative approach for the analysis of samples that are too complex to be resolved by a single separation method [15]. By directly coupling two different modes of separation, one can perform an initial separation of the fraction of interest, and then separate that fraction on a second column. This multidimensional separation technique can be easily automated, thereby reducing the analysis times of multi-component samples, and improving the reproducibility and detection limit of the analysis. Of the many possible combinations for multidimensional techniques, on-line HPLC/GC is well suited for the analysis of pesticide residues in complex matrices. In this mode, HPLC has been used to pre-separate pesticides from interfering components in corn [16] and butter fat [17]. Size exclusion chromatography (SEC) has also been incorporated into the HPLC pre-separation scheme for pesticide residues [1,18]. Multidimensional HPLC/GC was finally linked on-line with SFE, thus integrating all of the analysis steps that were necessary for the quantitative determination of chlorpyrifos insecticide residue in grass samples [19].

Although, there are difficulties in interfacing two chromatographic systems which use different mobile phases, the on-line interfacing of HPLC to GC has been made possible, by utilizing a narrow-bore packed HPLC column coupled via a rotary switching valve to a capillary GC column. An uncoated pre-column (retention gap) is normally used in between the columns to accommodate the change of the mobile phase from a liquid to gas [20]. This solvent evaporation technique requires a precise control of the operational factors such as the choice of liquid mobile phase, volume of transferrable fraction, and the evaporation temperature of the liquid, in order to

achieve maximum transfer and focusing of the analytes on the GC column.

Analogous to HPLC/GC, the combination of packed column SFC and capillary column GC has shown the potential application to complex matrices [21]. In SFC/GC, the problems associated with the evaporation of HPLC mobile phases before GC analysis, are avoided, since the SFC mobile phase is easily removed by decompressing into a gas. Furthermore, the transfer of large volumes of SFC effluent has no harmful effect on the GC separation. These inherent advantages of coupling SFC and GC were again realized, when the multidimensional chromatography was subsequently integrated with SFE [22]. In this configuration, SFC is used as an on-line cleanup step to remove co-extracted matters. The fraction containing pesticide residues is then subsequently diverted and separated by a capillary GC. The development and the applications of this instrumentation for the quantitative analysis of organo-chlorine and -phosphorus pesticide residues in fatty food samples, are presented in this report.

2 Experimental

2.1 Samples and Reagents

Samples of poultry fat containing incurred pesticides, were used in one experiment. The peritoneal fat sample contained the following incurred pesticides (heptachlor epoxide, dieldrin, and endrin). For blank and fortified samples, fat from a control chicken sample was used. Ground beef and lard were purchased from a local meat packing house. All samples, except lard, were prepared by blending them to a smooth paste in a food processor. Fortified samples were prepared by spiking these samples with an appropriate amount of the solution containing a mixture of either organo-chlorine or -phosphorus pesticides.

Analytical reference standards of pesticides were obtained from US EPA Pesticides and Industrial Chemicals Repository (Research Triangle, NC) and Chem Service (West Chester, PA). Individual stock solutions of organo-chlorine and -phosphorus pesticides were prepared in pesticide grade-hexane and -acetone, respectively, at 1 mg/ml concentration. Standard solutions for the spiking purpose and the working calibration were then prepared by serial dilution of composite stock solutions made from the individual stock solutions. The carbon dioxide used for SFE was obtained from Scott Specialty Gases (Plumsteadville, PA). For SFC, carbon dioxide – SFC/SFE grade (Air Products and Chemicals, Inc., Allentown, PA) was used.

2.2 Instrumentation for On-Line SFE/SFC/GC

The on-line SFE/SFC/GC analytical system was constructed by coupling two commercial instruments; a SFE-SPA system (Milton Roy Corp., Riveria Beach, FL) and a model 501 SFC unit (Lee Scientific, Salt Lake City, UT). Both systems required modification of their original configurations. In addition, an interface was fabricated and used to couple the two systems. A schematic diagram of the on-line system is shown in **Figure 1**.

2.2.1 Supercritical Fluid Extraction

For the SPA system, a 3.5 ml volume extraction vessel (Keystone Scientific, Inc., Bellefonte, PA) was used for up to 1 gram of fat samples. A three-position manually-operated valve (V1) provided three modes for the extraction loop operation: flushing of the loop, charging the loop with carbon dioxide, and closing the loop. A recirculating pump on the system maintained the flow in the closed loop extraction cycle. Sampling of the extract and injection into the SFC were accomplished by the pneumatic actuation of a six-port

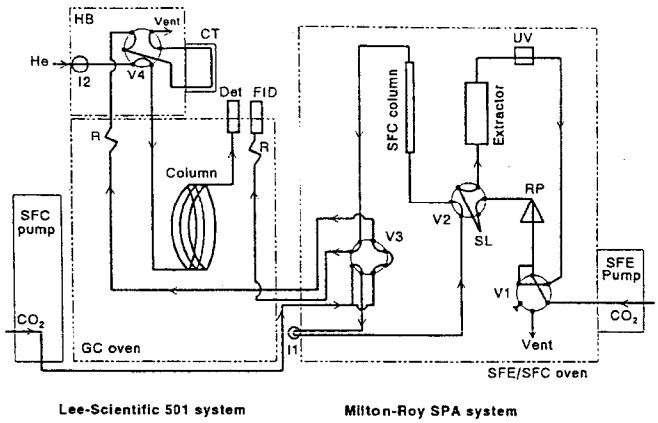

Figure 1
Schematic diagram of coupled on-line SFE/SFC/GC apparatus. V(1-4) = switching valves; R = flow restrictor; Det = ECD or NPD; RP = recirculating pump; SL = sampling loop; I1 = SFC injector; I2 = GC injector; CT = cryogenic trap; HB = heated block; He = helium.

rotary switching valve (V2) equipped with a 10 microliter volume sampling loop. Upon completion of extraction and sampling, the remaining supercritical solvent and dissolved compounds in the extraction vessel, were discharged through a vent port.

2.2.2 Supercritical Fluid Chromatograph

Although the SPA system was designed originally to perform SFE, SFC could be done in the SPA oven by installing a packed SFC column and redirecting the flow path of CO_2 mobile phase through the existing valve arrangement. For fractionation of pesticides from co-extracted fats, a Deltabond microbore column, 150 × 1 mm i.d. (Keystone Scientific, State College, PA), packed with 5 μm silica beads of covalently bonded octyl siloxane (C-8) stationary phase, was used. Supercritical carbon dioxide mobile phase was delivered by the syringe pump of the Lee Scientific 501 SFC system. The SFC effluent that exited the column was directed to either a flame ionization detector (FID), or a cryogenic trap, by using a 8-port switching valve (V3). Pressure restriction for the SFC was achieved by using approximately 25 cm length of fused silica capillary tubing of 15 μm i.d. (Polymicro Technologies Inc., Phoenix, AZ).

Fused silica capillary tubing of 50 μm i.d. served as transfer lines in the SFC. Low dead-volume unions (Valco Instruments, Co., Inc., Houston, TX) were used for these connections between restrictor and transfer line. The FID was operated at the temperature of 325°C. The flow rate (expanded) of SFC effluent was about 50 ml/min at a pump pressure of 200 atm and 50°C. An additional injection valve (Model CI4W.5, Valco Instruments Co., Inc., Houston, TX) with a 0.5 μl internal sample loop was installed to introduce fixed amounts of reference standards into the SFC column for instrument calibration.

2.2.3 Cryogenic Trap and Heated Valve Interface

A 6-port high temperature switching valve, V4 (Model C6WT, Valco Instruments Co., Inc.), mounted on a thermostated block was used for transferring heart-cut fractions of SFC to a custom-made cryogenic trap. The cryogenic trap consisted of a deactivated silica capillary tubing (20 cm × 0.25 mm i.d.) placed inside a thin-walled 1/8" stainless steel tube. The temperature inside the stainless steel tube was measured with a J-type thermocouple, and was manually controlled by cooling and heating with liquid carbon dioxide and nichrome resistance wire, respectively.

Pesticide analytes in the heart-cut fraction from a SFC separation were precipitated out and deposited inside the cold silica capillary tubing, while the depressurized carbon dioxide was vented through the heated 6-port switching valve (V4). The temperature of the cryogenic trap, which can be cooled down below –50 °C, was operated near 0 °C during the trapping cycle for the organo-chlorine and -phosphorus pesticides. Immediately after the trapping cycle, the analytes were desorbed into the GC column by rapidly heating the trap above 250 °C.

2.2.4 Gas Chromatography and Detectors

The final analytical separation of the pesticides fractionated in the SFC were carried out with a capillary gas chromatograph. For this purpose, the oven of the Lee Scientific Model 501 SFC was converted for use as a GC, and linked with the SFC through the cryogenic-trap interface. Detection of GC separated organo-chlorine and -phosphorus pesticides was conducted by switching between the electron-capture (ECD) and nitrogen-phosphorus (NPD) detectors, respectively. ECD and NPD were maintained at the temperatures of 300 and 250 °C, respectively. The separations of pesticides were achieved by using a fused silica capillary column (30 m × 0.25 mm i.d.) with 95% methyl + 5% phenyl siloxane stationary phase (J&W Scientific, Folsom, CA). Average linear velocity of helium carrier gas was 29.4 cm/s.

2.3 Operation of On-Line SFE/SFC/GC

Although both dynamic and static extractions can be performed, present studies were limited to the application of static extraction. In this mode, an extraction vessel was filled with a fixed volume of a supercritical fluid at a given temperature and pressure. Prior to transferring into an extraction cell, 0.5 to 1 gram fatty sample was mixed with either Hydromatrix or anhydrous sodium sulfate (1:1 and 1:8 by weight, respectively) to enhance the extraction. Concurrently, an internal standard, *o,p'*-DDD and Ronnel for analysis of organochlorine and -phosphorus pesticides, respectively, was added. The sample mixture was loaded into the extraction cell, and then heated to the desired temperature. Carbon dioxide was pumped into the extraction vessel by an auxiliary reciprocating pump, set to the desired pressure. Then the recirculation pump was activated to start the fluid recirculation in the closed loop mode.

At the end of the prescribed extraction-equilibration time (*ca.* 1 h), the sampling valve (V2) was switched to the inject position, which allowed the SFC mobile phase to sweep the sample extract into a packed SFC column. SFC was performed with carbon dioxide at 200 atm and 50 °C to separate pesticides from co-extracted fats. The retention behavior of these respective fractions was studied prior to sample processing, and it was found that 12 selected organo-chlorine pesticides eluted within 10 min under the above conditions. Fifteen minutes were required for complete elution of 6 selected organo-phosphorus pesticides.

The SFC eluent at the end of these corresponding time periods was directed onto the cryogenic trap by switching the 4-port valve (V3). Subsequently, the analytes were aspirated out of the restrictor as the fluid depressurized, and deposited inside the cold fused silica capillary trap at approximately 0 °C, while CO_2, due to lower boiling point temperature (*i.e.*, –78.5 °C), vented out. At the end of the trapping period, the 6-port high temperature valve (V4) was switched to the GC position, and the cryogenic trap was rapidly heated to 250 and 260 °C for organo-chlorine and -phosphorus pesticides, respectively. Helium carrier gas then swept the desorbed analytes into the capillary GC for final separation and detection. GC temperature program for the separation of organochlorine pesticides was started from 80 °C. After 2 min, the temperature was increased at 10°C/min to 180 °C, and then increased at 3 °/min to 250 °C. For the separation of organophosphorus pesticides, initial oven temperature was set at 100 °C for 2 min, which was subsequently increased at 10 °C/min to 200 °C, followed by 3 °/min ramp to 260 °C.

While the system was performing GC separation, the SFC column was cleaned and regenerated by rapidly increasing the carbon dioxide mobile phase pressure and maintaining it at the maximum pump pressure of 415 atm. Fatty materials retained on the SFC column were subsequently removed and discarded through the FID. Concurrently, the SFE loop was cleaned and prepared for the next sample by charging and discharging fresh CO_2 through an empty extraction vessel. Occasional flushing of the SFE loop with a liquid solvent (hexane or acetone), also ensured removal of any materials that were not soluble in supercritical CO_2.

3 Results and Discussion

3.1 Analysis of Organo-chlorine Pesticides

The on-line SFC separation of the pesticide residues in the supercritical carbon dioxide extract of a fortified chicken fat is shown in **Figure 2**. All of the organo-chlorine pesticides eluted within a short time period (10 min), while co-extracted lipids (mainly diglycerides and triglycerides) were retained on the C-8 packed-column. Similar separation behavior was noted for pesticides [23] and lipids [24] on the column with same type of stationary phase.

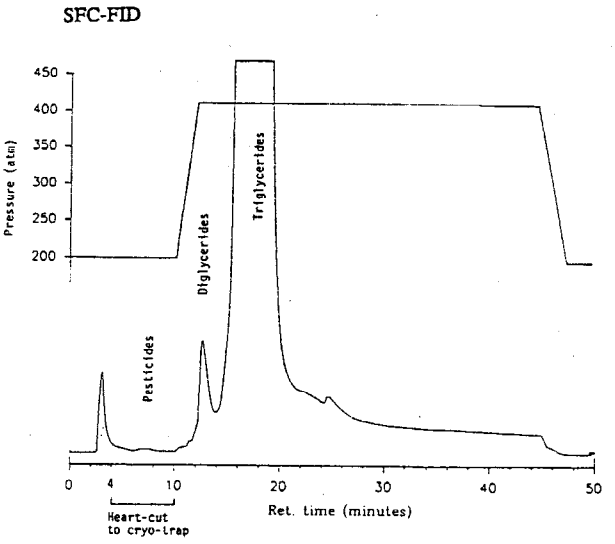

Figure 2
On-line SFE/SFC - flame ionization detection profile of chicken fat fortified with trace level of organochlorine pesticides.
*** Analytical conditions: One hour static SFE with CO_2 at 204 atm. and 50 °C; SFC with C-8 packed column and CO_2 at 50 °C; Cryo-trap at 0 °C followed by subsequent desorption at 250-260 °C.**

Subsequent cryogenic trapping/focusing of the pesticide fraction, followed by GC separation, yielded a chromatogram showing well-resolved pesticide peaks (**Figure 3**). By utilizing a sensitive electron capture detector, a number of extra peaks were observed in the chromatogram. However, these peaks eluted early and did not interfere in the pesticide quantitation above the minimum detectable levels given in **Table 1**. A blank sample, run immediately after

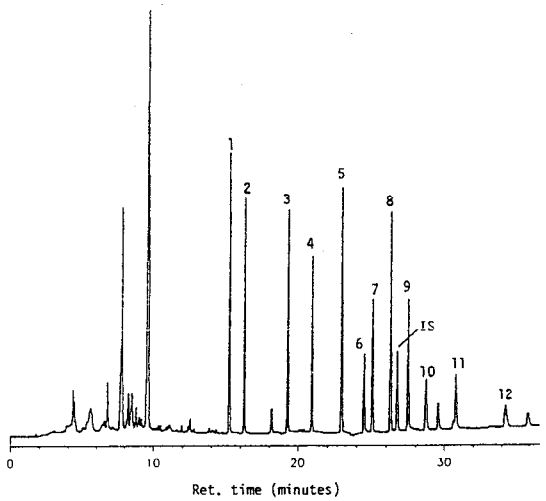

Figure 3
Chromatogram of organochlorine pesticides spiked at 0.5 ppm in chicken fat, analyzed by on-line SFE/SFC/GC-electron capture detector. (See Table 1 for the identification of the peaks; IS = internal standard.)

Table 1
On-line SFE/SFC/GC determinations of organochlorine pesticides from pesticide-fortified chicken fat.

Compound		Percent recovery ± SD[a]	MDL[b] (ppb)	FSIS/LDL[c] (ppb)
1	Hexachlorobenzene	85 ± 6	4	10
2	gamma-BHC	101 ± 8	8	10
3	Heptachlor	88 ± 7	8	10
4	Aldrin	98 ± 4	8	20
5	Heptachlor epoxide	102 ± 9	8	10
6	*o,p'*-DDE	88 ± 7	20	20
7	alpha-Chlordane	84 ± 4	12	100[d]
8	Dieldrin	116 ± 5	8	10
9	Endrin	125 ± 5	12	30
10	*p,p'*-DDD	93 ± 7	32	–
11	*p,p'*-DDT	85 ± 6	28	40
12	Methoxychlor	107 ± 8	68	150

[a] Standard deviation ($n = 5$).
[b] Minimum detectable level of on-line SFE/SFC/GC analysis ($S/N = 10$).
[c] Lowest detectable limit of the current appropriate methodology used by FSIS [25].
d) Sum of all chlordane isomers.

the analysis of a spiked sample, showed a negligible amount of carry over when the extraction vessel and the loop were thoroughly cleaned between runs.

A number of on-line SFE/SFC/GC analyses were performed for a chicken fat that was spiked with 12 organochlorine pesticides, at concentrations near the regulatory residue limits [25] of the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture. Average recoveries of the pesticides ranged from 84 to 125% (Table 1). Repeatability of the analysis is shown by low standard deviation (SD). The complete analysis including SFE of a sample was accomplished in less than 2 h, which was much shorter than the time required for the conventional off-line methodology using a liquid-based extraction. If SFE is configured to perform parallel extraction of a number of samples simultaneously, the total analysis time can be further reduced to about 1 h.

Poultry fat tissues containing incurred pesticides were also analyzed by both off-line and on-line methods, and the resulting chromatograms of the incurred residues are compared in **Figure 4**. Average concentrations incurred pesticide residues in chicken fat determined from eight repetitive analysis by the on-line system,

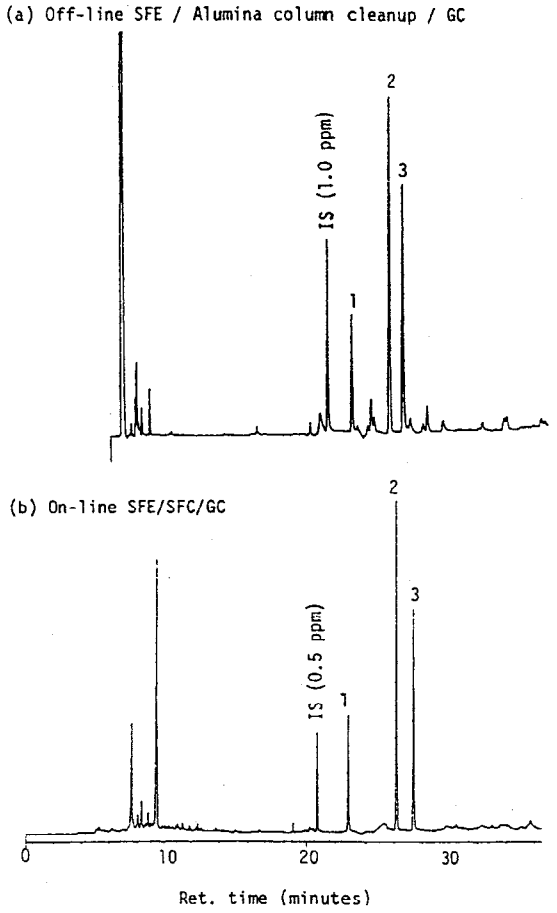

Figure 4
Chromatograms of incurred pesticides in chicken fat, analyzed by (a) off-line and (b) on-line methods. (See Ref. 26 for detailed analytical procedures for the off-line method.) IS = internal standard.
*** Mean pesticide concentrations determined by on-line method (8 repetitions): 1 = Heptachlor epoxide, 0.49 ± 0.02 ppm; 2 = Dieldrin, 1.75 ± 0.10 ppm; 3 = Endrin, 2.03 ± 0.04 ppm.**

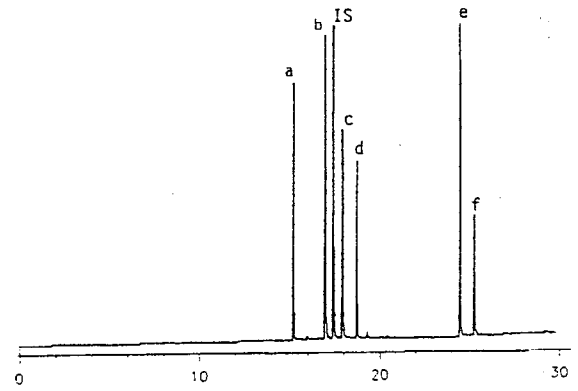

Figure 5
Chromatogram of organophosphorus pesticides spiked at 0.5 ppm in chicken fat, analyzed by on-line SFE/SFC/GC-nitrogen/phosphorus detector. (See Table 2 for the identification of the peaks; IS = internal standard.)

Table 2
On-line SFE/SFC/GC determinations of organophosphorus pesticides from pesticide-fortified fatty food samples.

Compound		Percent recovery ± SD				Detection limit	
		Chicken fat[a)]	Ground beef[b)]	Lard[b)]	Hydromatrix[b)]	MDL[c)] (ppb)	FSIS/LDL[d)] (ppb)
a	Diazinon	88 ± 4	91 ± 2	90 ± 1	80 ± 1	8	100
b	Methyl parathion	104 ± 4	94 ± 5	103 ± 3	97 ± 0	10	100
c	Fenitrothion	99 ± 5	94 ± 4	101 ± 1	95 ± 1	14	100
d	Chlorpyrifos	87 ± 1	85 ± 1	96 ± 0	76 ± 1	14	50
e	Ethion	80 ± 1	79 ± 3	82 ± 1	84 ± 1	6	100
f	Carbophenothion	78 ± 5	85 ± 6	82 ± 6	84 ± 5	22	30

a) $n = 8$.
b) $n = 4$.
c) Minimum detectable level of on-line SFE/SFC/GC analysis ($S/N = 10$).
d) Lowest detectable limit of the current appropriate methodology used by FSIS [25].

were: 0.49 ± 0.02 ppm of heptachlor epoxide, 1.75 ± 0.10 ppm of dieldrin, and 2.03 ± 0.04 ppm of endrin. Considering the long elapsed time since the previous study, as well as the use of calibration standards from the different origins, these values compare favorably to the concentrations (0.56 ppm, 2.33 ppm, and 2.00 ppm, respectively) determined previously by an off-line SFE method [26]. In addition, no significant difference between these pesticide concentrations and the values obtained by a conventional technique of liquid-based extraction, was observed.

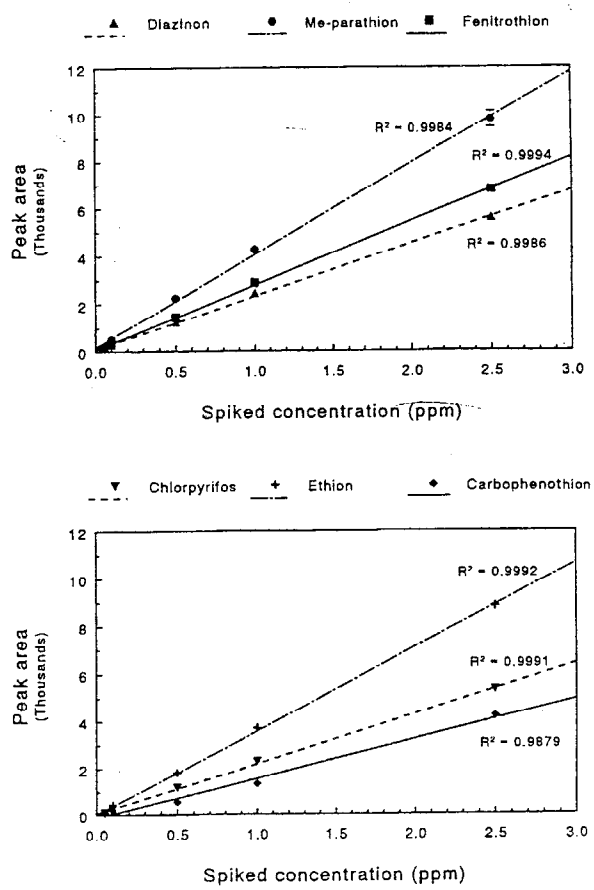

Figure 6
On-line SFE/SFC/GC calibration curves for organophosphorus pesticides in chicken fat.

3.2 Analysis of Organo-phosphorus Pesticides

Operating at conditions similar to those for the organochlorine pesticides, the on-line SFE/SFC/GC system was used to analyze six organophosphorus pesticides from fatty samples. An example of the excellent chromatographic results obtained for a pesticide fortified chicken fat is shown in **Figure 5**. No extraneous components that responded to the nitrogen-phosphorus detector were visible at the 500 ppb detection level for the organophosphorus pesticides. The measured minimum detectable levels (6 to 22 ppb) by the on-line analysis were well below the lowest detectable limits, which can be obtained by FSIS methodologies (**Table 2**). Pesticide residues were quantitatively recovered from three fatty sample matrices (pesticide-fortified chicken fat, ground beef, and lard) and an adsorbent (Hydromatrix). If needed, extraction efficiency can be improved for the analytes that had incomplete recovery, by adding a small amount of co-solvent during the extraction. Addition of approximately 3% methanol to CO_2 yielded higher extraction efficiency for these relatively polar pesticides [27].

The overall efficiency of the on-line SFE/SFC/GC analysis were determined by comparing the amount of recovered pesticides to the reference standards, introduced directly into SFC/GC. However, more accurate quantitation is realized, when the recovery is calculated based on the calibration curves of SFE/SFC/GC, instead of SFC/GC. Linear regression analyses and corresponding correlation coefficients indicated that the analytical technique by on-line SFE/SFC/GC was linear with respect to pesticide concentrations ranging from 0.05 to 2.50 ppm in chicken fat matrix (**Figure 6**).

4 Conclusions

The described on-line SFE-chromatographic system, where SFE has been coupled with SFC and GC, allowed efficient analyses of organo-chlorine and -phosphorus pesticide residues from gram-sized sample matrices, such as chicken fat, ground beef, and lard. Utilizing supercritical CO_2, the method proved considerably faster and less laborious, than the conventional analytical procedures based on liquid extraction. The analytical results obtained from the on-line procedure, compared favorably with those obtained by conventional extraction, cleanup, and analysis methods. Future studies will include the examination of alternative SFC columns, thereby extending the technique to other classes of pesticides.

Note

Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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